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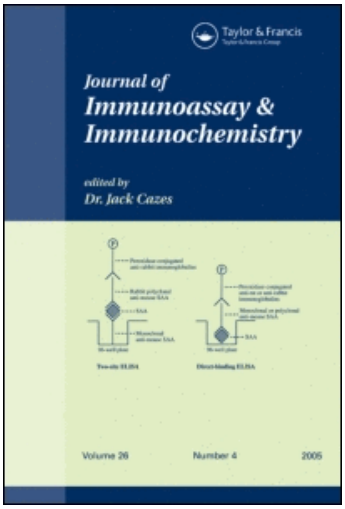

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A Rapid and Sensitive Heterogeneous Immunoelectrochemical Assay Using Disposable Electrodes

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A RAPID AND SENSITIVE HETEROGENEOUS IMMUNOELECTROCHEMICAL ASSAY USING DISPOSABLE ELECTRODES

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Key words: Immunosensor, electrochemical sensor, immunoelectrodes

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ABSTRACT

In this novel enzyme-tagged immunoelectrochemical assay, disposable carbon felt discs serve both as electrodes and as the heterogeneous solid phase. Antibodies are immobilized on the carbon felt via a diaminoalkane-biotin-avidin-biotin bridge. Alkaline phosphatase is used as a label. Bound antibodies are monitored by following the electro-oxidation of aminophenol, produced enzymatically from p-amino-phenyl phosphate by the immobilized alkaline phosphatase at the electrode surface. A model system designed for determination of mouse IgG concentration yielded a calibration curve ranging from 10pg/ml to 100μg/ml. This assay can be performed rapidly and a single determination completed within 20 minutes. The system is useful also for rapid quantitation of a small number (~80 organisms per ml) of bacteria.

INTRODUCTION

During the last decade, numerous attempts were made to combine the specificity of antibody-antigen interactions with the high sensitivity, wide dynamic range and simplicity of electro-

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analytical methods. These attempts led to the development of immunoassays, which show great promise in fields such as medical diagnostics (1).

Electrochemical immunoassays involve the coupling of an electroactive moiety to an antigen or an antibody either directly (2-4) or via an enzyme (5-14). In electrochemical enzyme immunoassays, usually an antigen or an antibody is tagged with an enzyme and the enzymatic reaction is monitored by a potentiometric or an amperometric electrode. The amplification attained by the enzyme catalysis is particularly advantageous in the detection of very low concentrations (15). Recently, several reports have described the use of electrode surfaces both as the immunological solid phase and as the electrochemical detector (16-18).

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In a previous publication (19) we described preliminary results obtained with an immunosensor based upon the enzymatic reaction of alkaline phosphatase with its substrate p-aminophenyl phosphate. In that system, glassy carbon served both as the solid phase in the immunorecognition reaction and as an amperometric electrode that oxidizes the aminophenol formed in the enzymatic reaction. Glassy carbon electrodes were also used in a heterogeneous immunoassay using glucose oxidase as an enzyme label(16-17). Huet et al. (20) recently showed, by using rotating disc electrodes made of glassy carbon, that mass transfer to the solid phase consisting of antibodies immobilized to the glassy carbon is a key step in such heterogeneous immunoassays.

Although the results obtained by us and others using glassy carbon looked promising, we encountered several problems: Quantification of the results was difficult because the electrodes were not uniform, considerable time was required for completion of the reaction, the dynamic range of the assay was narrow (only one order of magnitude) and the precision of determination was inadequate. We assumed that most of these problems could be attributed to the insufficient and highly variable amounts of antibody adsorbed to the solid phase during the first step of the assay. We therefore attempted to improve the antibody-binding capacity of the electrode surface by varying both the nature of the carbon surface and the number of antibody-binding sites. These efforts led to the development of the novel immunoassay described in this paper.

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MATERIALS AND METHODS

Materials

1,6-Diaminohexane (hexamethylenediamine, HMD) was obtained from Fluka AG (Germany). 1-Cyclohexyl-3-(morpholino-ethyl) carbodiimide metho-p-toluenesulfonate (CCD) and all other materials were obtained from Sigma (USA) unless otherwise indicated. Formalin-fixed Staphylococcus aureus was obtained from Sigma. p-Aminophenyl phosphate (APP) was synthesized as

described previously (21). Carbon felt sheets (RVG 1000) were obtained from Le Carbon Lorraine (France). Carbon felt discs (5 mm diameter) were cut from the carbon felt sheets.

Immobilization of Antibodies

Antibodies were immobilized on the carbon felt discs either by adsorption or via a biotin-avidin-biotin bridge as described below. The discs were then stored in a desiccator at 4°C.

Immobilization by adsorption: Discs were incubated in 10 ml PBS containing 10 µg/ml of anti-mouse Ig antibodies (Sigma M8642) for 16 hours at room temperature with shaking.

Binding of antibodies to carbon felts via a biotin-avidin-biotin bridge: Discs were immersed in a solution of 1 M HMD and 500 mg/ml of CCD in water, final pH 5.0 (with HCl), and incubated for 16 hours at ambient temperature with shaking. After thorough washing with PBS the discs were immersed in 1 ml of 100 mM Na-phosphate buffer pH 8.0. Following the addition of N-hydroxy-succinimide biotin (NHS-biotin) dissolved in dimethylformamide (DMF) to a final concentration of 1 mg/ml, the mixture was incubated for 16 hours at ambient temperature with shaking. The discs were then washed well with PBS, placed in 1 ml of PBS containing 50 µg/ml avidin, and incubated for 10 minutes with shaking. Finally, the discs were placed in 1 ml PBS containing biotinylated anti-mouse Ig antibodies (diluted 1/250 from a

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commercial preparation), incubated for 10 minutes with shaking and washed extensively with PBS.

Immunoelectrochemical Assay

Prior to use the carbon felt discs were mounted on a special housing made of a teflon cylinder containing a concentric platinum wire and a teflon cap with a stainless steel net (Fig. 1). This assembly served both as a heterogeneous phase for antigen (analyte) capture and as the working electrode in the amperometric measurement, and is referred to henceforth as the antibody electrode. The assay was performed with 5-10 electrodes in parallel.

The immunoelectrochemical assay consists of three steps:

1. Antigen capture: Each of the antibody coated electrodes was immersed in 1 ml of analyte solution in PBS and rotated at about 1000 rpm for 5 minutes (unless otherwise specified), followed by gentle washing in water.

2. Conjugate binding: The electrodes were then introduced into 1 ml of conjugate solution (Sigma anti-mouse IgG antibodies, coupled with alkaline phosphatase (AP) and diluted 1/250 in PBS solution containing 2% Tween 20 and 1% BSA) and rotated at about 1000 rpm for an additional 5 minutes.

3. Electrochemical determination of enzyme activity: After being gently washed in water the electrodes were transferred into the

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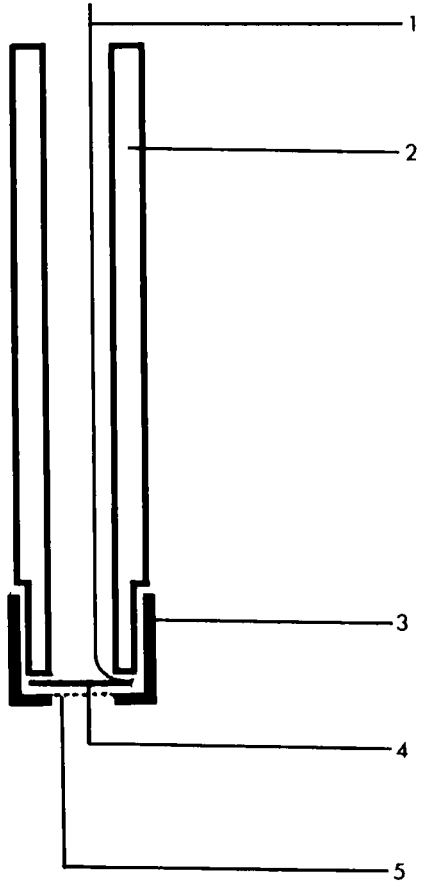

FIGURE 1. Schematic layout of the carbon felt electrode configuration. 1- platinum wire, 2- teflon cylinder, 3- teflon cap, 4- carbon felt disc, 5- stainless steel net.

electrochemical cell containing 5 ml of substrate solution (3.7 mg/ml of APP in 50 mM Na_2CO_3, pH 9.6) and rotated at about 500 rpm. A potential of 0.22 V was applied between the antibody electrode and a calomel electrode (Radiometer K-401 Copenhagen,

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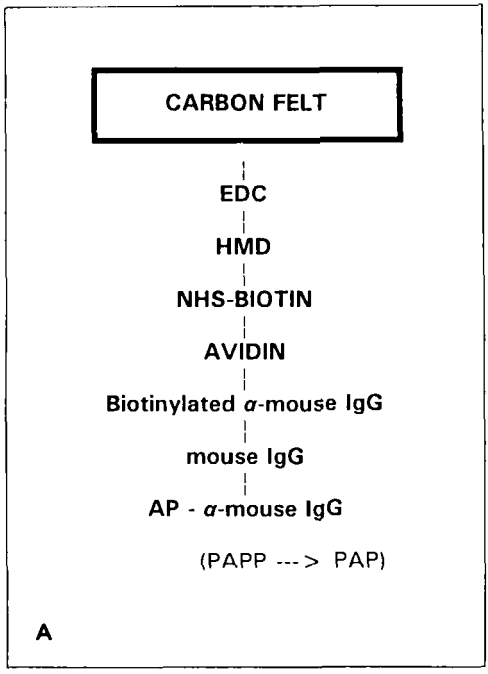

FIGURE 2. Scheme showing the immunoelectrode structure.
a. Antibody electrode for the determination of mouse IgG.
b. Antibody electrode for the determination of S. aureus.

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Denmark), and current production between the working electrode and a counter electrode (platinum mesh) was measured. A computerized electrochemical system described earlier (22) was employed for current reading and signal averaging. Measurement was automatically repeated until readings were stabilized, which usually occurred within 1 minute. A complete scheme of the immunoelectrodes is presented in Fig. 2a.

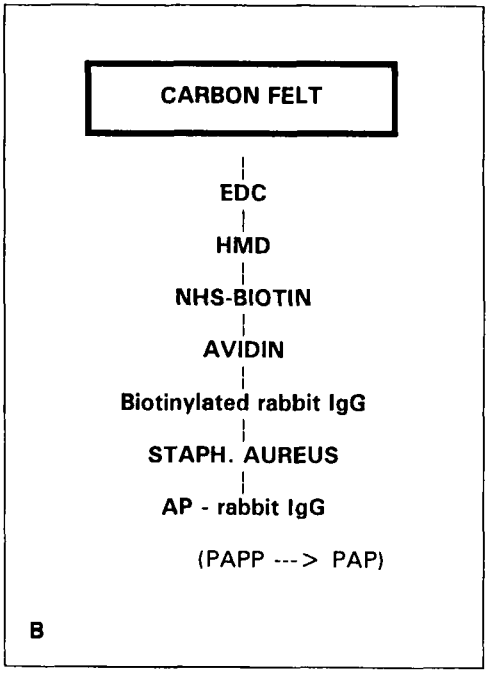

FIGURE 2B

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Detection of Staphylococcus aureus

A general scheme for detection of the bacteria is presented in Figure 2b. Antibody electrodes were prepared with immobilized rabbit IgG (affinity-purified rabbit anti-mouse Ig antibodies) via the biotin-avidin-biotin bridge, as described above. The electrodes were then rotated for 5 minutes in PBS suspensions of different S. aureus at different concentrations, washed with PBS, and rotated for 5 minutes with an AP-conjugated rabbit antibody (rabbit anti-mouse IgG) in PBS blocking solution containing 1%

BSA and 2% Tween 20. The electrochemical signal was measured following washing as described above.

RESULTS

Immobilization of Antibodies

The ability of the carbon felt discs to serve both as the heterogeneous phase in an enzyme immunoassay and as an electrochemical detector was studied first in a model sandwich system. Antibodies (anti-mouse IgG) were immobilized on the carbon felt discs by physical adsorption. The discs were then used for the determination of various mouse IgG concentrations according to the procedure described above. The results are presented in Figure 3 (lower curve).

Although a calibration curve was obtained the amounts of active antibody immobilized by this procedure were relatively small, resulting in a narrow dynamic range of the assay, low limit of detection (LOD), low sensitivity and insufficient reproducibility between different discs. We therefore substituted covalent immobilization for adsorption, in an attempt to increase the amount of antibody immobilized on the carbon felt.

Initially, we tried to immobilize the antibodies on the carbon felt using the carbodiimide bridge preceded by an electro-oxidation step to form a carboxylic group on the carbon (23).

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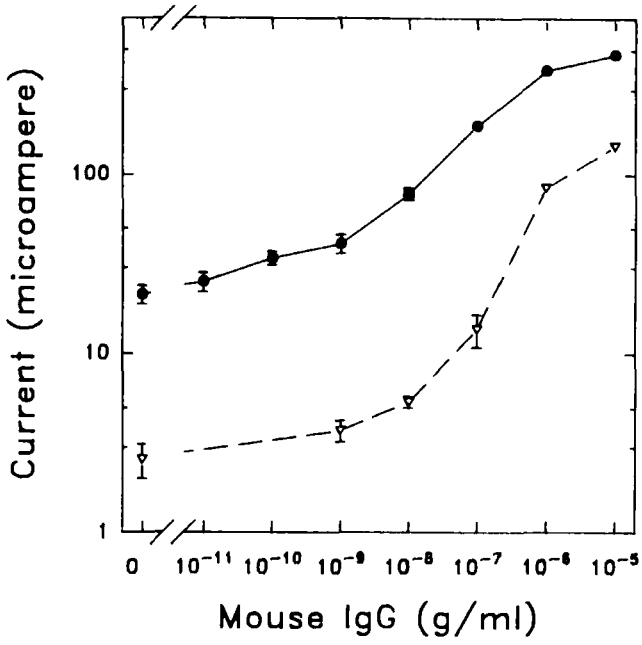

FIGURE 3. Immunoelectrochemical determination of mouse IgG by carbon felt electrodes
Lower curve: First antibody immobilized by adsorption.
Upper curve: First antibody immobilized via HMD-biotin-avidin-biotin bridge.

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This technique, which had proved successful in binding of antibodies to glassy carbon electrodes, was found here to produce high background currents that interferred with the electrochemical measurement. These currents could be eliminated if the preliminary preoxidation step was excluded. However, neither the assay sensitivity nor amount of bound antibody as detected by radiolabeling showed any advantage over the physical adsorption technique..

Several reports have documented the importance of a spacer arm in ligand coupling (24). An adequate spacer arm promotes the effective utilization of active sites with a minimum degree of blockage and allows for flexibility and mobility of the antibody molecule as it protrudes into the solvent. Accordingly, we introduced spacers between the carbon felt and the first antibody molecules by employing the following procedures:

1. Binding via diaminoalkane: HMD was bound to the carbon by incubation in buffer containing high concentrations of HMD and CCD. Under these conditions CCD mediates the binding of HMD to free carboxyl groups on the carbon felt surface. Next, the aminated carbon felt discs were activated by immersion in glutaraldehyde (GA) solution and the antibodies were then immobilized on the GA-activated carbon discs.

2. Binding via biotin-avidin-biotin: Carbon felt discs were biotinylated by incubation with biotin hydrazide and with CCD, which mediates the binding of hydrazide groups to free carboxyl groups on the carbon. The biotinylated discs were then reacted with avidin and the avidin-coated discs were in turn reacted with biotinylated antibodies. Use of the biotin-avidin-biotin bridge is a well documented strategy in immunochemistry (25,26).

3. Binding via diaminoalkane and biotin-avidin-biotin: Carbon felt discs were aminated with HMD, as described in procedure 1 above. The aminated discs were biotinylated by reaction with NHS-biotin, followed by reaction first with avidin and then with biotinylated antibodies, as described in procedure 2 above.

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Although antibodies were immobilized by procedures 1 and 2, the amounts of immobilized active antibodies did not exceed those obtained by physical adsorption, as judged by the enzymatic activity obtained from the electrocatalytic current in a standard assay system. However, a significant increase in the enzymatic activity of the electrodes was accomplished by procedure 3, i.e. by a combination of the diaminoalkane spacer and the biotin-avidin-biotin bridge. The resulting high electrocatalytic currents led to a more satisfactory limit of detection (LOD), an increased dynamic range and an improved sensitivity compared with results obtained with all other procedures used for immobilization of the antibodies (Fig. 3 upper curve).

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Effect of Electrode Rotation on Rate of Analyte Binding

The antibody-antigen reaction in solution or at the solid-liquid interface is not usually diffusion limited. However, reactions at the solid-liquid interface of artificial surfaces are limited in practice by mass transport (27). In most heterogeneous immunoassays (with the exception of immunofiltration assays, (28)), incubation times of at least 1 hour at ambient or elevated (37°C) temperature are necessary for complete interaction of antigens with the immobilized antibodies.

A rotating electrode causes a flow of the fluid tangential to the electrode surface, resulting in the formation of a diffusion

layer of constant thickness. It was previously shown that the flow of antigen molecules to the electrode surface is controlled by the parameter D/d, i.e. the diffusion coefficient of the antigen, and by the thickness of the diffusion layer (20).

Rotation of an electrode made of carbon felt, which is non-rigid and has a surface area in a finite volume of the antigen solution, effects a rapid contact between the solution and the surface. Moreover, the immobilized antibodies are at a high enough concentration to efficiently capture a high proportion of the antigen molecules. Consequently, with the rotating electrodes incubation times are significantly reduced, the immunological reactions are completed within 3-5 minutes. We selected a speed of about 1000 rpm as a standard for analyte and second antibody binding. The exact effect of the rotation speed on the assay sensitivity is currently under investigation.

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Effect of Temperature on Electrode Signal

Theoretically, temperature might influence the immunoelectrochemical measurement in three ways : a. By affecting the kinetics of the binding of the analytes to the immobilized antibody and the binding kinetics to the conjugate; b. affecting the enzymate activity, and c. by affecting the electrochemical kinetics. Initial experiments indicated however that when a rotating electrode is used, under the hydrodynamic

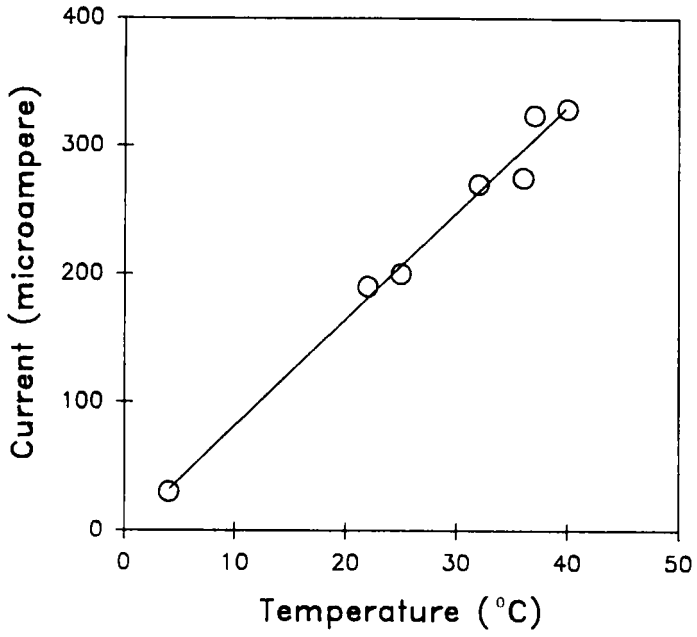

FIGURE 4. Effect of temperature upon the electrochemical signal. (See text for details)

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conditions specified in the preceding section, the kinetics of ligand binding are not temperature dependent.

We then examined the effect of temperature on the measured signal. Since the aminophenol formed was determined at 0.22V, which is in the diffusion controlled region of the oxidation of the aminophenol (19), and since the substrate was in excess, any effect of the temperature should be related to the enzymatic activity. Accordingly, the increased enzymatic activity observed at around 37°C (Fig. 4) is expected, since the AP used for conjugation to the antibody was prepared from bovine intestinal

mucosa. Generation of the electrochemical signal at 37°C helped to enhance the precision of the assay as well as increasing the measured signal.

Detection of Bacteria

Detection of S. aureus was selected as a model system for testing the ability of our immunoelectrochemical assay system to detect bacteria. This bacterium was selected since the protein A on its cell surface binds immunoglobulin.

Using an assay format identical with that used for detection of mouse Ig, we were able to detect the presence of as few as 80 bacteria per ml in 20 minutes (Fig. 5).

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DISCUSSION

The predominant type of immunoassay used today is a colorimetric, heterogeneous enzyme immunoassay, which is available in a variety of different forms. Disadvantages of these include their narrow dynamic range, which usually necessitates testing of several dilutions of the sample, and the relatively high cost of the ELISA readers. The common alternatives, fluorescent and luminescent enzyme immunoassays, possess a wider dyanamic range, better LOD, and greater sensitivity than the colorimetric assays,

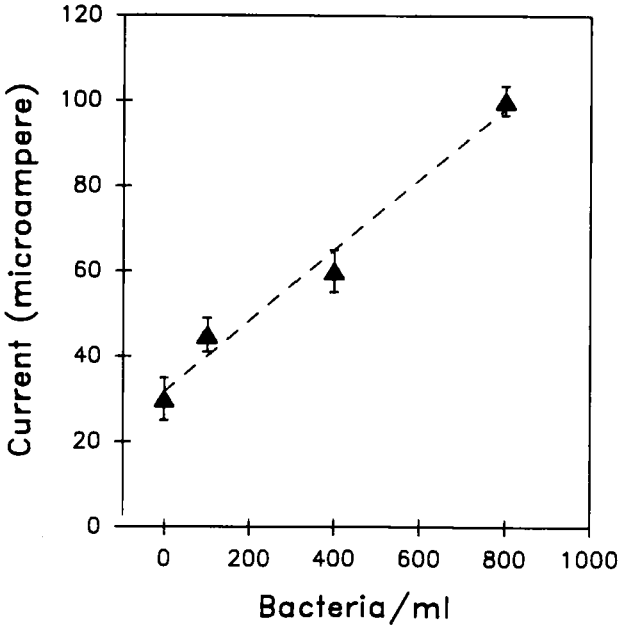

FIGURE 5. Detection of S. Aureus by the immunoelectrochemical assay system.

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but fluorescence and luminescence readers are even more expensive than the colorimetric ELISA readers. The last decade has seen a growing interest in the development of rapid and sensitive immunoelectrical techniques. The various potentiometric and amperometric immunoelectrodes described in the literature indicate than an electrode-based immunoassay could offer a simple and cheap alternative approach. Amperometric immunoassays involve somewhat more instrumentation than potentiometric ones, but possess much higher sensitivity (29). In particular, amperometric enzyme immunoassays that involve a specific enzyme reaction resulting in

current generation offer not only a heightened sensitivity, but also a high dynamic range.

In this work we have used a specific model electro immunoassay system and compared the results with those obtained previously (19) using the same antibodies, the same antigen, and the same enzymatic and electrochemical reactions. The advantages of the present system are as follows: :

1. The use of carbon felt instead of glassy carbon, coupled with covalent immobilization of antibodies via a spacer, results in a solid phase possessing a high capacity and hence an increased dynamic range, spanning from 10 pg/ml to 100 µg/ml. The system can therefore be conveniently used for the determination of a large number of analytes, with minimal dilution of the test sample. The high current improves the assay sensitivity and also makes the system more robust.

2. The time needed to perform the test was reduced from 1-3 hours to 5-20 minutes. For semi-quantitative determination a total assay time of less than 5 minutes is adequate.

3. The use of a rotating electrode enables the system to deal with relatively large volumes. This effectively enhances the sensitivity of certain assays (e.g. urine analysis, water testing), where the amount of test material is not a limiting factor.

4. Unlike glassy carbon, which must be cleaned after each use, the carbon felt disc (the sensing part of the carbon felt electrode) not only has a very large effective surface area, but is also cheap and disposable.

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The LOD obtained with the present assay system is 10 pg/ml. Although this sensitivity is adequate for most clinical immunoassays in current use, it is not remarkable compared with other heterogeneous assays. For example, Kitamurao et al. (30) reported an LOD of 0.3 pg/ml for IL-2 using a fluorescent label, and Bronstein et al. (31) reported an LOD of 0.7 pg/ml for alkaline phosphatase using a chemiluminescent enzyme immunoassay. Jenkins et al. (32) recently described an immunoelectrochemical system employing the same enzyme label and substrate as those used by us and which, with proper background control, reportedly detected 7.5 pg/ml of mouse IgG. In this system, however, an electrochemical sensor was used to monitor phenol production by alkaline phosphatase adsorbed to polystyrene, and the phenol was quantitated by liquid chromatography monitored by means of an electrochemical system. Although much more complicated than ours, the system described by Jenkins (32) suggests that with further refinement of our system we might be able to improve the LOD. Our assay system, therefore, appears to be potentially capable of much greater sensitivity and could thus complete with the most sensitive immunoassays described to date. A factor still limiting the sensitivity is the high background, which we are currently attempting to reduce.

An enzyme-linked immunosensor capable of detecting protein A-bearing S. aureus in pure cultures and in food samples has been described by Mirhabibollahi et al. (33). This assay relies on the amperometric detection by an O_2 electrode of O_2 generated from

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H_2O_2 by catalase linked to the secondary antibody. The sensitivity attained was 10^4 cfu/ml. The assay was performed by using antibody-coated membranes in a glass container, and was therefore difficult to automate. The electrochemical determination was time-consuming and required the separation of the reaction mixture from air oxygen. An improved amperometric immunosensor, based on the detection of phenol produced by alkaline phosphatase, could detect 10^3 cfu/ml (34). In that immunoassay microtiter plates were used as the heterogeneous phase, and as a result the procedure was relatively time-consuming (2-3 hours). In the present work utilization of the same surface for both the immunological recognition and the electrochemical detection results in a more rapid and more sensitive detection of S. aureus. Initial measurements of the concentration of S. aureus using this system showed that it was possible to detect ~80 organisms per ml. Subsequent trials (data not shown) indicated that as few as 5-10 organisms per ml could be reproducibly detected in a 10-minute assay and that the assay could be performed directly in food samples. These results further support our belief that this assay could still be improved to the point where the LOD for protein analytes is lower than 1 pg per ml. Our assay could then favorably compete with the most sensitive immunoassay available today. The advantages of our immunoelectrochemical assay system, namely, the ease and rapidity of performance, low price, adequate LOD and extended measurement range, can be expected to make it a popular choice for the detection of protein analytes and bacteria.

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